Identification of ultraviolet radiation induced gallic acid and caffeic acid formation in palm juice (*Borassus flabellifer*) by HPLC & mass spectra technique

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Abstract Palm juice, a common-cheap-antioxidants rich natural plant juice has been investigated for optimizing the effect of UV-radiation on the antioxidant activity using a DPPH free radical scavenging activity method. In this study separate set of samples of raw palm juice has been treated with 365 and 254 nm UV-lights (UVL) respectively for different exposure time. When exposed for 15 min with 365 nm UVL induces concentration factor of caffeic acid, whereas, 254 nm UVL induces gallic acid accumulation, but overall antioxidant activity was higher for 365 nm UV-radiation. Caffeic acid and other polyphenol compounds are increased by 5.5 ± 0.5 % than normal palm juice, observed after irradiation with 365 nm UVL. Even after the exposure of UV irradiation for 15 min, did not affect on peptide bond modification of protein molecules present in palm juice, therefore a green effect of UVL is explored for the effective increase of antioxidant activity.

Keywords Antioxidant activity · DPPH · HPLC · Palm juice · UV irradiation

Introduction

Plants have evolved different defensive systems against environmental stresses such as salinity, drought, temperature, pollutants, metal toxicity or ultraviolet radiation which generates highly reactive oxygen species (ROS) [1]. This higher level of UV radiation may damage DNA, proteins and lipids, in biological cell [2], UV radiation also evokes oxidative stress although the mechanism of ROS generation in UV-B irradiated plants is not known [3, 4]. These ROS (superoxide radical, hydrogen peroxide, hydroxyl radical and other free radicals) is extremely reactive and cytotoxic [5]. Plants have evolved protective mechanisms by various enzymatic and non enzymatic antioxidants to minimize these deleterious reactions. These enzymatic and non enzymatic antioxidants are included superoxidedismutase, catalase, ascorbic acid etc., and their activity is greatly enhanced by UV-radiation [4, 6–8].

Hydroxycinnamic acids and flavonoids are two major classes of antioxidants and their concentrations are measurably increased in response to solar irradiation [9]. The salad crop *Gynura bicolor* had a lower level of flavonoid when it was grown in a UV-B light protected glass house, compared with open field condition [10]. In lettuce (*Lollo Rosso*), whenever the effect of light on the phenolic content has been investigated, leaves exposed to higher light levels have been found to have higher concentration of flavonoids. Hydroxycinnamic acids are commonly found in the esterified form with quinic acid. The major dietary hydroxycinnamic acid is caffeic acid (3,4-dihydroxycinnamic acid) which is found in food mainly as chlorogenic acid (5-O-caffeoylquinic acid) because of its conjugation with quinic acid. Caffeic acid, trans-3-(3,4-dihydroxycinnamic acid) prophenic acid is most commonly frequently occurred in fruits, vegetables, cereals, legumes and in

beverage of plant such as wine, tea and coffee [2]. The caffeic acid has ability to inhibit the formation of hydroxyl radicals in the in vitro condition under UVA irradiation. The inhibitory effect of caffeic acid in the formation of hydroxyl radicals in the reaction mixture under UVA irradiation is seems to occur through a novel antioxidant activity [11].

We report herein a paradigm shift in the concept of UVL use and introduce it for the purpose of increasing antioxidant activity in natural food materials in a in vitro condition. Therefore this study evaluates the effect of UV radiation on the polyphenol compounds of raw palm juice and the changes observed in the concentration of the polyphenols and their antioxidant activity depend on the exposure time, during the irradiation period.

Materials and methods

Chemicals

HPLC grade methanol and HPLC grade water purchased from Merck India. (+) Catechin hydrate obtained from Sigma–Aldrich, USA. Gallic acid purchased from SD fine Chem Ltd India.

Sample collection

Palm juice (*Borassus flabellifer*) was randomly collected from local traders in rural areas of South 24 parganas District, West Bengal, India. Palm juice was harvested after 12 h of collecting time in a mud jar during tapping process using bamboo tube. After that the bottles of palm juice were kept in the refrigerator. During transportation time (2–3 h) it was carried with ice bag and brought to our laboratory. It was preserved at −50 °C in an ultra low temperature freezer (Model C340, New Brunswick Scientific, England).

Ultraviolet irradiation effect

In a 100 ml beaker 50 ml of raw juice was taken and kept in a UV chamber, the distance from sample to light source was adjusted to 15 cm. The juice was treated with 254 nm (4.881 eV) and 365 nm (3.396 eV) wavelengths of UVL for different time of incubation period for 10, 15, 20 and 30 min. The antioxidant activity, total phenolic content and total flavonoid content were measured in UVL treated sample centrifuged at 2,500 rpm for 20 min.

Determination of DPPH radical scavenging activity

The effect of the sample on DPPH radical was estimated according to the procedure described by Brand-William et al. (1995) [12] was followed. The sample (0.1 ml) was added to freshly prepared 3.9 ml of DPPH (100 μM) in ethanol and incubated for 45 min. After incubation, the absorbance of sample was measured at 515 nm by Spectrophotometer (Model 2800, Hitachi, Japan). The 0.1 ml ethanol solution and 3.9 ml of DPPH solution were used as control and only ethanol was considered as blank. The inhibitory percentage of DPPH was determined according to the following Eq. (1):

$$\text{Scavenging effect (\% of inhibition)} = [1 - (\text{absorbance}_{\text{sample}}/\text{absorbance}_{\text{control}})] \times 100 \quad (1)$$

Determination of FRAP radical scavenging activity

Prepared the FRAP reagent by following a process- (a) 300 mM acetate buffer at pH 3.6; (b) 10 mM TPTZ in 40 mM HCl; (c) 20 mM $FeCl_3$ $6H_2O$. The working FRAP reagent was prepared by mixing a, b, c in the ratio of at 10:1:1. 100 μl of the sample was mixed 3 ml of working FARP reagent and measured at 593 nm [13]. The result are expressed by FRAP value in mmol of $FeSO_4 \cdot 7H_2O$/100 g of sample.

HPLC analysis for polyphenol detection

The samples were analyzed on C18 (4.6 × 250 mm) 5 μm, 300 Å reverse phase column in HPLC (JASCO, MD-2015 Plus multi wavelength detector) at room temperature by using a mobile phase of water/methanol/acetic acid (87:8:5) under isocratic mode with flow rate 1 ml/min. The compounds were detected at 280 nm. The chromatography peak was identified by comparing the retention time of the sample with respect to reference standard [14].

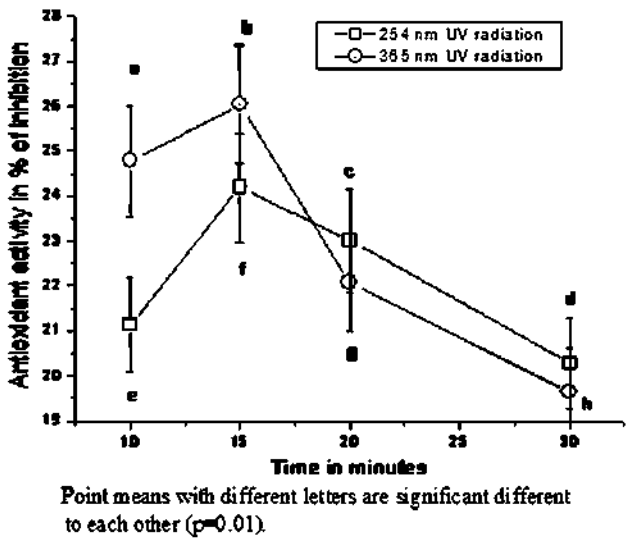


Fig. 1 Effect on antioxidant activity of raw palm juice with time at 254 and 365 nm wave length of UV light

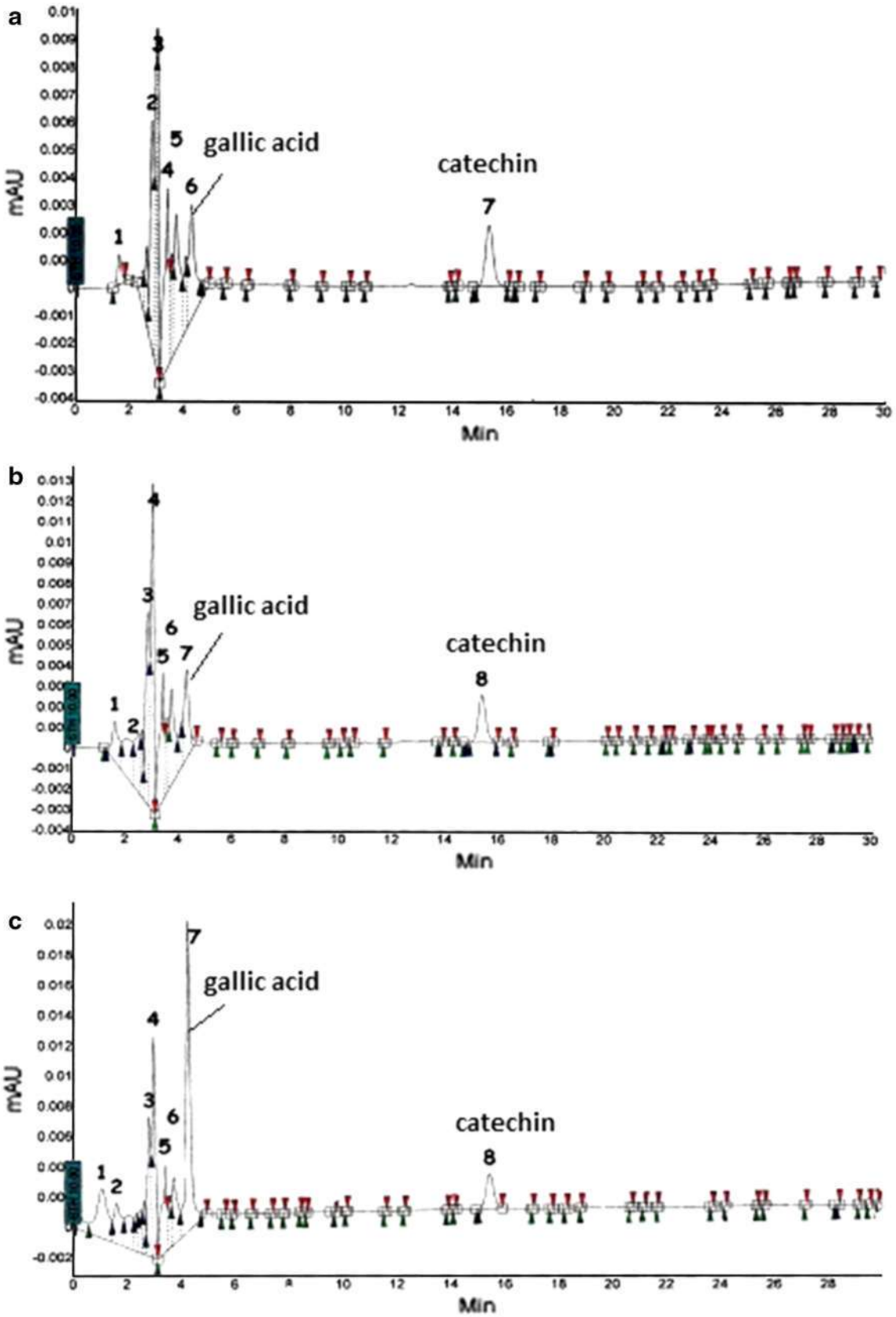


Fig. 2 HPLC-PDA analysis of palm juice for **a** control, **b** 365 nm UV treated, **c** 254 nm UV treated detected at 280 nm. *Peaks* identification: 6, gallic acid; 7, catechin (**a**); 7, gallic acid; 8, catechin (**b**, **c**)

Mass spectroscopy analysis

The UV treated juice and untreated raw juice were analyzed by the mass spectroscopy system. The effects of the UVL on the antioxidant compounds were analyzed with a quadrupole time-of flight micro mass spectrometer (Q-TOF–MS TM, Waters) with electrospray ionization (ESI) in ESI–MS mode and data acquisition was done in positive and negative mode.

SDS-PAGE analysis

The SDS-polyacrylamide gel electrophoresis was performed on 4 % (w/v) stacking and 10 % (w/v) resolving gels as described by Laemmli (1970) [15] in a mini-protein tetra cell equipment (Bio-Rad, USA) with four samples i.e. raw juice, palm wine, and 365 and 254 nm UVL treated (for 15 min) juice. The gel was stained by Comassive Brilliant Blue R250 and destined by glacial acetic acid, methanol and water (10:50:40) till visible the bands.

Statistical analysis

Statistical release 8 software (Statsoft, USA) was used for data analysis. All experiments were performed in triplicates and data was presented as mean ± SD for three replicates in each sample. The Fisher least significance test was used to check equality of variances and one way ANOVA was used to estimate the statistically significant difference ($p \leq 0.05$).

Results and discussion

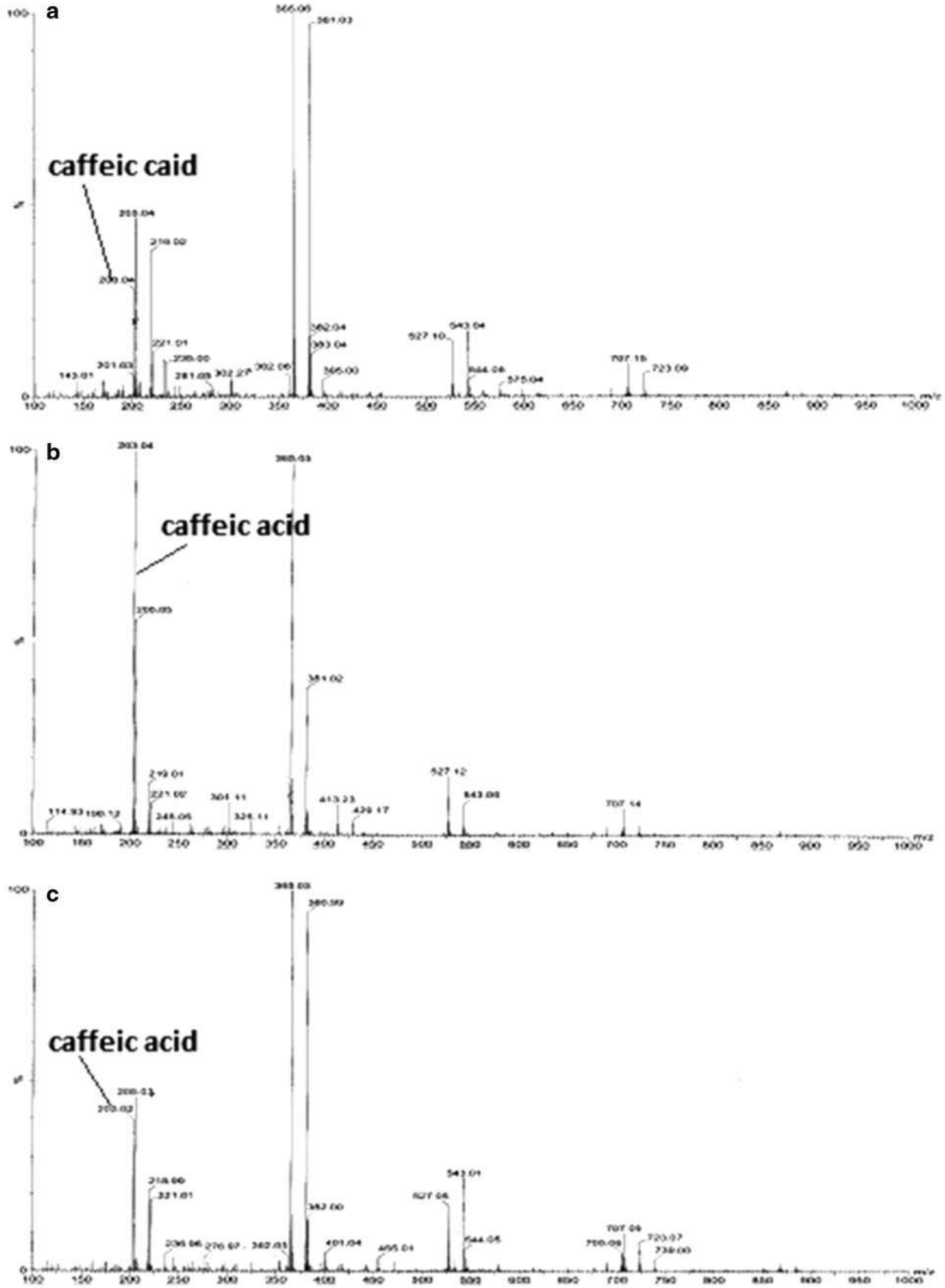
Effect of UV irradiation on antioxidant activity of raw palm juice

UV radiation enhanced the antioxidant activity of the raw palm juice; it is when treated with different wavelengths of UVLs for different incubation time that induced the antioxidant activity of the raw palm juice. 365 and 254 nm UVLs are treated with palm juice for 15 min incubation time and the DPPH radical scavenging activities were 26.04 % of inhibition (42.03 ± 1.2 mg/l of catechin equivalent) and 22.68 % of inhibition (36.61 ± 1.6 mg/l of CE) (calculated from Eq. 1) as shown in Fig. 1. The FRAP values of 365 and 254 nm UVLs treated palm juice for 15 min incubation time were expressed in 35.15 ± 2.8 and 30.13 ± 2.4 mmol respectively. In DPPH and FRAP method, the antioxidant activities of untreated raw palm juice were 20.54 % of inhibition (33.81 ± 0.9 mg/l of CE) and 16.12 ± 1.4 mmol respectively. UV radiation increases accumulation of the phenolic acid, flavonols, flavones, and total flavonoids compounds [16]. Alothman et al. 2009 [17] reported that the effect of UVL on fresh cut fruit like banana, pineapple and guava, the antioxidant capacity, total phenolics and flavonoids were increased due to UVL. In effect of the UVL plant increase their polyphenol compounds with the use of depolymerization and dissolution of cell wall polysaccharides [18]. This might also hold true in our present observations, wherein UVL might have facilitated that type depolymerization or synthesis of polyphenol compound with the use of palm juice containing carbohydrate or other natural compounds.

Fig. 3 Positive ion electrospray mode of MS analysis of palm juice ▶ for **a** control, **b** 365 nm UV treated, **c** 254 nm UV treated. The identified mass spectrum of *m/z* 203.04

Exposure of the UVL raises phenolic acid level and also increases total phenol, cyanidine glycoside, quercetin, luteolin phenolic acid of lettuce [19]. So rather than in vivo study, we also used it in vitro condition and from this study, it has been observed that 365 nm UVL was more effective for increasing antioxidant activity as well as total phenolics and flavonoid content rather than 254 nm UVL. It has been reported that 254 nm UVL treatment on fresh cut tropical fruits reduced total phenolics and vitamin C [17]. We know that palm juice contain ascorbic acid therefore, it is also one of the reasons that 254 nm treated juice has shown lower antioxidant activity rather than 365 nm treated juice.

The longer wavelength (365 nm) of UVL for 15 min incubation showed more antioxidant activity than the shorter wavelengths (254 nm) UVL (Fig. 1) because the time of exposure was an important factor for UV treatment. It was reported that the *trans* resveratrol can undergo isomerization to the *cis* form when exposed to UVL at 360 and 254 nm. This *trans* resveratrol change to *cis* resveratrol by exposure to 360 nm UV radiation for 20 min whereas 30 min exposure is needed for the same isomerization at 254 nm UVL. The antioxidant activities are dependent on the isomers of the compound i.e. *trans* isomers of resveratrol are more powerful antioxidant compared to *cis* isomers [20]. Previous study by Hernandez et al. 2007 [20] performed experiments with standard resveratrol in ethanol solution but we have used the natural palm juice which contains so many polyphenols and other compounds, so there is a higher possibility of this type of isomerization reaction. Change in the functional group of the polyphenols in UV treated palm juice at different treatment time is probably the reason for different antioxidant activity in it compared to the various exposure time treated palm juice. Alothman et al. 2009 [17] also reported that the exposure time of UVL was strongly correlated with antioxidant activity. For guava, differences in the antioxidant capacity values were insignificant for the 0, 10, and 20 min


a
caffeic caid
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treatments; therefore this statement also supported our observing result.

HPLC analysis was performed to ascertain the antioxidant compounds were either formed or altered due to exposure of UV irradiation. The HPLC data showed that the formation of the peaks of 365–254 nm UV treated juice were different from the control juice (not treated by UV light). The peaks showed in HPLC chromatogram of the control juice between 2 and 5 min were less intense, whereas UV treated juices showed distinct sharp peaks. Moreover, the identified peaks 6 and 7 (Fig. 2a) and peaks 7 and 8 (Fig. 2b, c) were for gallic acid and catechin respectively. These two peaks were coincided with the retention time of standard polyphenol i.e. gallic acid and catechin. The gallic acid is highly influenced by 254 nm UVL, it has been reported that when tomato was treated with 254 nm UVL, the gallic acid was a major phenolic compound in tomato fruit and significantly increased the contents of gallic acid due to 254 UVL [21]. Therefore this peak height was higher rather than UVL 365 nm and control. Gallic acid shows low retention time, it belongs to benzoic acid group; it has three OH groups and shows highly polar nature and antioxidant activity. Therefore low retention time of the peaks of the control and UV treated juice are because are benzoic acid group containing polyphenols and high polar nature. Dziedzic et al. 1983 [22] also reported that the position of the hydroxyl group in phenolic compound plays an important role in response to their chemical nature. Hydroxylation of the B ring is largely responsible for the antioxidant activity of the compound [22]. Phenolic compounds with the same basic structure may be induced when treated with UVL to change the position of additional OH, methyl or other functional group and create difference among the analytes in overall polarity and solubility. For e.g. the hydroxy benzoic acid show same basic structure but have varying polarity due to position of hydroxyl group, and polarity is decreased in the order of—3′,5′dihydroxy benzoic acid, 2′,5′dihydroxy benzoic acid, 4 hydroxy benzoic acid, benzoic acid and salicylic acid respectively. Therefore due to the UVL treatment, there is change in position of functional group of benzoic acid ring containing polyphenol in palm juice. The same reason is applicable for UV treated palm juice where its peak height and retention time differ from non treated palm juice. But the different type of UVLs affected low to the catechin, due to this reason if we compare the two UV treated juice at 254 and 365 nm it could have the same peak height but they were could be different from control peak hight of catechin (Fig. 2b, c).

All the unknown peaks were not identified by HPLC method. For identification of the peaks MS analysis has been done. Three different samples (control juice, 365 and 254 nm UV treated juice) show that some of the peaks of same mass have different heights (Fig. 3a, b, c). The peak of *m/z* 203.04 was identical in mass with M + Na of our known antioxidant compound (caffeic acid). The peak height of *m/z* 203.04, varied from control juice and 254–365 nm UV treated juice. The peak height of *m/z* 203.04 was 100 % for 365 nm treatment whereas for control and 254 nm treated juices, the peak heights were 30–62 % respectively. The MS analysis data have confirmed that new mass of compounds was synthesized and their peak heights varied due to UVL exposure. One of the identified mass of caffeic acid which is an important antioxidant compound, whose height was maximum in 365 nm UVL, treated juice and hence it also showed highest antioxidant activity. This result also supported by Cantos et al. 2000 [23] reported that Napolean table grapes were irradiated by 340 and 254 nm UVL and found that various polyphenol compounds were affected by UV irradiation and caffeic acid was one of them. All this statement are supported that 365 nm UV treated juice showed more antioxidant activity than the 254 nm UV treated juice and control.

Effect of UV irradiation on protein component of raw palm juice

SDS-PAGE was performed to attribute a comparison of protein profile in between UVL treated and untreated raw palm juice. SDS-PAGE analysis (Fig. 4) shows that the three samples such as raw palm juice, 254 and 365 nm UV treated palm juice were shown in lane no. 1, 2 and 3 that were within 20–30 kDa band.

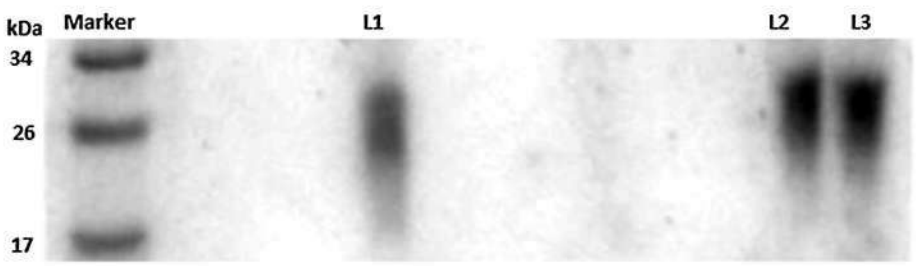

Fig. 4 SDS-PAGE analysis. Marker: pre-stained molecular weight marker; *L1* raw palm juice, *L2* 254 nm UV irradiated raw palm juice, *L3* 365 nm UV irradiated raw palm juice

In the SDS-PAGE analysis, we found that UV irradiation only affects photochemical compounds but does not affect peptide bonds present in protein complex since no such additional bands of high and low molecule weight proteins were found (lane 2 and lane 3) in SDS-PAGE profile even after UV exposure. This SDS-PAGE analysis can justify only on the basis of the molecular weight of the protein molecules, which was not affected by UV exposure. Therefore the control palm juice and UVLs treated palm juice containing proteins were of the same molecular weight (20–30 kDa). From the above experimental data it might be concluded that 15 min UVL exposure time was not engulfed for changed their molecular weight of protein molecules but other physical or chemical changed was not revealed by the SDS-PAGE.

Conclusion

UVL has been proved to be have multidimensional applicability in sterilization process, mutation, isomerism of chemical compound or inducing the plant phytochemicals by in vivo condition. But we have focused on the use of UVL for the synthesis of natural compound i.e. caffeic acid, gallic acid from palm juice by in vitro process. 365 nm UVL possesses greater potential effect to induce polyphenol compounds of which caffeic acid has been identified. On the other hand 254 nm UVL enhances the formation of gallic acid. But overall antioxidant activity is highest induced in 365 nm UV treated palm juice. In all cases UVL (365 and 254 nm) for 15 min incubation time has not affected to the palm protein molecules. Therefore the molecular weight of the protein molecules have not been changed. In future this research work can be extended to find out more detailed mechanism of UV inducing processes.

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